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ABSTINENCE-INDUCED OXIDATIVE STRESS IN MODERATE DRINKERS IS IMPROVED BY BIONORMALIZER.

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ABSTRACT

The aim of this investigation was to study the oxidative phenomena taking place in the early recovery phase after alcohol withdrawal. Further, the effects of a novel natural antioxidant, i.e. Bionormalizer (BN), in such clinical setting was studied. Forty-six alcoholics with moderate drinking habits (daily ethanol intake: >80g - <120g) were enrolled and divided in two groups given either placebo or 9g/nocte of BN by mouth for one week. Patients agreed to stop alcohol intake and daily blood sampling was obtained for routine tests and to check plasma and erythrocyte level of MDA, SOD, GPX and hydroperoxide level. Groups were comparable as for initial biochemical parameters. BN prevented the early increase of plasma TBARS observed in placebo group enabling a near-to-normal level of plasma and erythrocyte MDA already on the fourth day. BN also prevented the significant drop of erythrocyte GPX and the transient decrease of plasma SOD observed in placebo group. Despite alcohol withdrawal, plasma lipid hydroperoxide remained significantly elevated in placebo group but this phenomenon was rapidly improved by BN. BN is able to significantly prevent free radical-mediated lipoperoxidative changes occurring soon after alcohol withdrawal while fastening the recovery mechanisms.

Key words: alcohol withdrawal, antioxidant status, free radicals, Bionormalizer.

INTRODUCTION

Alcohol oxidation is able to cause a wide number of metabolic and pathologic changes in the liver and lipid peroxidation is an important mechanism during alcohol-induced liver damage (1-3). In particular, microsomal hydroxyl radical products in the liver during alcohol oxidation have been shown to increase serum level of malonildialdehyde (4). This is a stable product resulting from free radical-mediated peroxidation of polynsaturated fatty acids of cell membranes (5). On the other hand, the concentration of antioxidant may be inadequate in alcohol-abusers, expecially when highly reactive oxidant species are generated in large amounts (6). Chronic alcohol consumption enhances the activity of microsomal ethanol oxidazing system. Further, Lieber et al. (7) have also shown that such increased metabolic activity may persist independently of the constant presence of alcohol intake. Thus, this phenomenon would perpetuate a pro-oxidative condition in the body of alcoholics while on abstinence.

The purpouse of this study was to address the issue of the oxidative phenomena taking place in the early recovery phase during alcohol withdrawal in alcoholics while testing a novel natural japanese health food, i.e. Bionormalizer, endowed with potent free radicals-scavenging property, as ascertained by recent studies (8-10).

MATERIALS AND METHODS

Patients.

Forty-six alcoholics (34 males and 12 females; age range: 34-55 with moderate drinking habit (>80g - <120g ethanol daily for at least 3 years) were enrolled in the study. None of the patients had clinical or biochemical evidence of acute liver disease and were negative for HBV and HCV infection. All patients underwent a carefull personal interview to establish ethanol consumption and drinking behavior. A detailed diet-history method was carried out and nutrient intakes were calculated by using a standardize food-composition table. None of the patients was feverish or malnourished.

On entering the study, patients were selected by their willingness and practical compliance in stopping alcohol intake, at least throughout the study period. Their reliability was checked by blood ethanol measurement (spectrophotometric method based on NAD reduction) as well as by other routine liver tests. Patients were also instructed not to perform any strenuous physical exercise during the observation period.

Patients were randomly divided into two groups (23 patients each) which were matched as for gender, age and nutritional status. On the day patients stopped alcohol, they were allocated into one of the following groups:

a) no treatment to serve as control. To assure phychological compliance, patients were blindly given 9g/nocte of flavoured sugar by mouth devoid of any antioxidant or vitamin for 1 week;

b) Bionormalizer, in a form of a whitish sweet powder, 9g/nocte by mouth for 1 week.

No vitamin supplement was allowed during the study period. As healthy control, 15 age- and gender-matched subjects teetoller were considered.

Patients underwent a daily one-week follow-up with clinical examination, dietary interview and biochemical tests, as outlined below.

Methods.

Blood samples were taken using EDTA as anticlotting agent and immediately refrigerated in ice. The plasma was separated by centrifugation at 4°C and the leucocyte layer was promptly removed by aspiration.

Plasma TBARS was assayed by the method of Yagi (11) modified by adding 0.01% butylated-hydroxytoluene to the colouring solution to avoid the formation of TBA reactive molecules during the analytic procedure. Tetra-ethoxy-propane served as a standard source of malonildialdehyde which is a TBARS. All samples were processed in duplicate. The remaining erythrocyte was washed in %ml ice cold phosphate buffered saline pH 7.4 and sedimented for five minutes at 1500rpm. Aliquots of 0.5ml each of the packed erythrocyte were added to 1ml 50% trichloroacetic acid. Malonildialdehyde in the erythrocyte extracts was assayed by HPLC analysis, according to Esterbauer et al. (12). Briefly, 0.2ml of packed eruthrocyte were thoroughly mixed with the same volume of ice cold acetonitrile in 1.5ml Eppendorf tubes. Following 20 minutes of extraction in ice the sampleswere centrifuged for one

minute at 13.000rpm and 20μl of supernatant was analysed by HPLC (Lichrospher column, Merck, Darmstadt, Germany) and acetonitrile/30mM TRIS buffer, pH 7.4 (1:9 vol/vol) as eluent. The effluent was monitored at 270nm wavelength and the malonildialdehyde peak in the chromatogram was identified by comparison with that of free malonildialdehyde standard freshly prepared by the hydrolysis of malonildialdehyde-bisdiacetal in 1% sulphuric acid solution. Glutathione peroxidase and superoxide dismutase activities (SOD) of plasma and erythrocyte were determined in a centrifugal analyzer (Hoffman La Roche, Switzerland), as described by Guèmori et al. (13). Plasma lipid hydroperoxides, mostly phospholipid hydroperoxides, were estimated by hemoglobin catalysed oxidation of 10-N-methylcarmoyl-3,7-dimethylamino-10-H-phenothiazine after treatment with phospholipase D, according to Ohishi et al. (14). Cumene hyroperoxide was used as standard. Plasma phsospholipid was determined by an enzymatic kit (Boehringer Biochemia, Mannheim, Germany). The plasma content of alpha-tocopherol was measured by HPLC analysis, according to Burton et al. (15). Briefly, plasma aliquots (1ml) wre mixed with 1ml 100mM sodium dodecylphosphate solution in water, 2ml absolute ethanol and 1ml n-heptane and shaken for one minute. Following 15 minutes of extraction in the ddark, the heptane phase was separated by centrifugation and 50μl aliquots were used for the HPLC assay. Values were read by a fluorescence detector set at 296nm excitation and 325nm emission wavelength.

Statistical analysis. Data were analyzed using the non-parametric Mann-Whitney U-test and correlations were tested using Kendall-Tau test for non-parametric data.

RESULTS

Two patients on Bionormalizer were withdrawn from the study because one admitted few light-alcohol beverage on the fifth observation-day and another missed one blood check-up. The two groups of patients considered were comparable as for baseline routine liver function tests and smoking habit. Smokers were nearly 50% in both groups and gamma-glutamyl-transpeptidase was the most significant biochemical abnormality (over four-fold increase). Comparable pattern and quantity of drinking habit was recorded in both groups, daily wine intake being the most common attitude. No statistical difference appeared between the groups as for dietary components during the study period. In particular, regarding dietary vitamin intake, no significant difference was recorded between the two groups of alcoholics and the healthy control group. Table 1 shows the baseline data regarding the antioxidant status of the two groups of alcohol abusers. At the entry, as compared to healthy control, both groups showed significantly lower level of plasma alpha-tocopherol, plasma ascorbic acid, plasma selenium and erythrocyte GPX. On the other

hand, levels of serum and erythrocyte MDA and plasma levels of GPX, SOD and lipid hydroperoxides were significantly higher in alcoholics. No significant difference appeared between the two groups of patients in the initial status of oxidative stress. Routine liver tests, body weight or smoking habit were not affected by Bionormalizer treatment. However, as compared to placebo group, patients treated with Bionormalizer reported a trend increase of appetite, as acertained by specific dietary interview.

Figure 1 shows the effect of Bionormalizer on plasma MDA. After alcohol cessation, a significant early increase ($p<0.05$) of MDA was observed in patients under no treatment. Such increase was self-limited by returning to normal values by the seventh day. However, concomitant supplementation with Bionormalizer perevented such phenomenon ($p<0.01$) by enabling a steady state of plasma MDA and a return to values comparable to healthy control already on the fourth day. From erythrocyte MDA assay it appeared that during the observation period in placebo group a trend decrease was observed. However, no significant change occurred. As compared to pre-withdrawal values, this parameter in Bionormalizer group showed a significant decrease from the fourth day ($p<0.05$) (figure 2). Either plasma GPX and erythrocyte GPX in both groups showed a decrease during abstinence period, irrespective of the treatment. However, while at the end of the study period erythrocyte GPX level in placebo group was significantly lower than in pre-withdrawal period ($p<0.05$), this did not occur in Bionormalizer group (figure 3). SOD plasma level was significantly higher in both drinker groups either at

the entry and at the end of the study ($p<0.05$). Such parameter showed a transient but significant decrease following alcohol withdrawal in the placebo group ($p<0.05$). Conversely, Bionormalizer brought about an inverse slope with three-day observation level significantly increased, as compared to placebo group ($p<0.05$). Irrespective of the treatment, erythrocyte SOD did not show any significant change over the abstinence period (figure 4). Plasma lipid hydroperoxide level in alcoholics was significantly higher than in controls ($p<0.01$) and no change occurred in the placebo group during the observation period. However, starting from the first observation-day, a significant decrease was recorded in Bionormalizer (7.1 ± 1.3nmol/ml vs 9.8 ± 0.9nmol/ml, $p<0.05$) group with near-to-normal levels at the end of the study (3.2 ± 1.3nmol/ml).

DISCUSSION

Our baseline data agreed with the established knowledge of reduced serum level of selenium, alpha-tocopherol and ascorbic acid in alcoholics. Further, only a marginal recovery of these parameters was observed after one-week abstinence period, irrespective of the treatment. During the metabolism of alcohol to acetaldehyde and its further metabolic process by cytochrome P4502E1, toxic free radical metabolites are generated. Such products trigger lipoperoxidative reactions which, on their turn,

are implicated in the pathogenesis of liver damage (16-18). Consistently high level of cytochrome activity (up to ten-fold) as well as enhanced liver mithocondrial sensitivity to lipid peroxidation may persist for a while after cessation of alcohol intake in case of chronic exposure to ethanol (7) and particularly in the early withdrawal stage (19). However, there have been only scanty reports on the effect of alcohol withdrawal on the oxidative status (6). Indeed, by our closely monitored data it appeared that early in the course of abstinence, oxidative stress indicators such as plasma lipid hydroperoxide, erythrocyte and plasma MDA in placebo group maintained a level comparable to pre-withdrawal period and thus, being significantly higher than in healthy control. In particular, it was of interest to note that plasma MDA in this group showed a transient but significant further increase within the third observation-day. Despite controversies still exist in the literature, in accordance with Lecomte et al. (6), erythrocyte SOD was unaffected by either alcohol ingestion and abstinence, irrespective of the treatment administered.

Plasma measurement of glutathione is an unreliable indicator of hepatic stores. Further, chronic ethanol feeding accelerates the turnover of GSH. Also Cu/Zn-SOD enzyme, which is the first line of defense against oxigen-derived free radicals, is rapidly induced following oxidative stress. However, such a tentatively compensatory measure might be insufficient, given the critical balance of the overall antioxidant system in alcoholics, thing that also appeared in our patients. Accordingly, baseline and one-week observation level of plasma SOD and glutathione in both

groups of drinkers was significantly higher than in healthy control. However, in placebo group a trend decrease was observed during the first three days with a statistically lower value on the third day. These data suggest that a pro-oxidative condition with an avid consumption of SOD and glutathione still takes place once alcohol ingestion is stopped. On the other hand, the present investigation suggests a beneficial effect of oral administration of Bionormalizer, a natural free radicals-scavenger, on the acute phase of recovery from oxidative stress following long-lasting moderate alcohol abuse. Erythrocytes in patients with alcohol-related liver disease may express an enhanced susceptibility to oxidative stress due to derangement of structural membrane lipids and to metabolic abnormalities. Accordingly, a reduced half-life of erythrocytes has been shown in patients with moderate/severe liver cirrhosis. Under Bionormalizer treatment, erythrocytes were far less susceptible to peroxidative changes (significant reduction of erythrocyte MDA and partial sparing of the erythrocyte GPX depletion). If the susceptibility of erythrocytes to peroxidative stress reflects that of other cells, and of hepatocytes in particular, then alcoholics would benefit from increased dietary supplementation of effective natural free radicals-scavengers, such as Bionormalizer. The optimal protection in the cell against oxidative damage is likely to be a result of a complex and balanced interrelationship between several antioxidant systems which lay on constitutive and newly synthetized forms. Further, limitations have to be considered in the present study as for number of patients and period of

observation. Nonetheless, the modulatory effect exerted by Bionormalizer seems of worthwhile interest when taking into account that very recent experimental data have questioned the preventive effect of o-tocopherol on ethanol-induced hepatic lipoperoxidative changes by showing, on the contrary, that high doses promote concomitant oxidative reactions in the liver with undesirable metabolic consequenses (20). Doses of Bionormalizer up to three times those ones employed in the present study have not shown any such untoward effect (Marotta F. unpublished data).

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table 1

ANTIOXIDANT STATUS
at the entry

	group 1 (23)	group 2 (21)
P-o-tocopherol (μmol/L)	24.6 ± 9.7	24.4 ± 10.6
P-ascorbic ac. (μmol/L)	1.2 ± 13.8	22.0 ± 16.5
P-selenium (μmol/L)	0.79 ± 0.2	0.68 ± 0.6
S-MDA (μmol/L)	4.2 ± 0.7	4.4 ± 0.6
P-SOD (U/L)	33.6 ± 2.7	32.5 ± 2.6
P-h-peroxide (nmol/L)	9.7 ± 0.7	10.1 ± 1.2
P-GPX (U/L)	699.1 ± 68.9	689.3 ± 55.4
RBC-MDA (nmol/ml)	4.6 ± 0.6	4.4 ± 0.8
RBC-GPX (U/g Hb)	34.7 ± 8.1	33.7 ± 10.1
RBC-SOD (U/L)	22.3 ± 4.3	20.9 ± 3.6

Control group significantly differred as for the following:

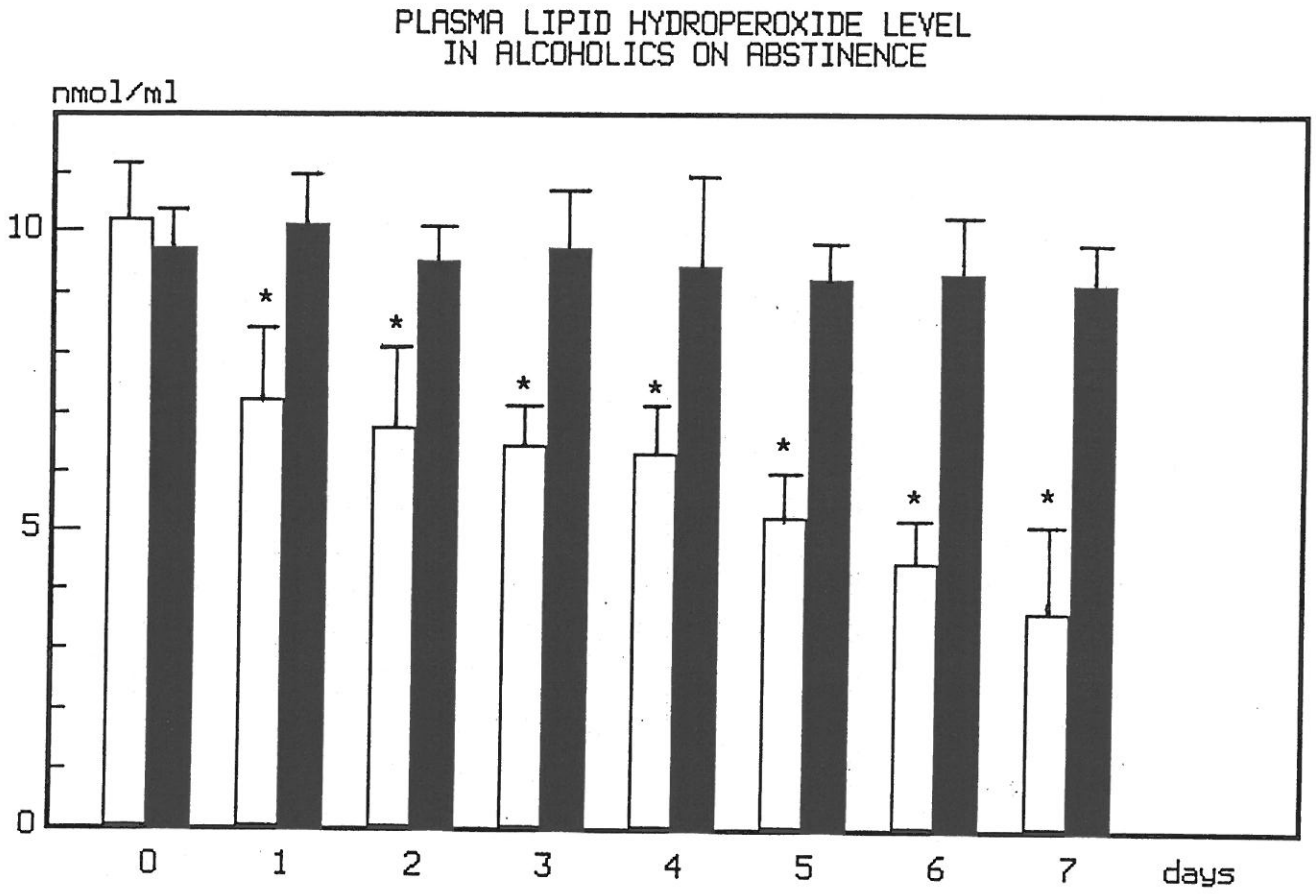
P-o-tocopherol (μmol/L)	: 32.4 ± 6.1	(p<0.01)
P-ascorbic acid (μmol/L)	: 38.9 ± 11.2	(p<0.001)
P-selenium (μmol/L)	: 0.94 ± 0.2	(p<0.05)
S-MDA (μmol/L)	: 2.6 ± 0.6	(p<0.05)
P-SOD (U/L)	: 27.2 ± 10.4	(p<0.01)
P-h-peroxide (nmol/ml)	: 2.3 ± 0.5	(p<0.001)
P-GPX (U/L)	: 631.2 ± 99.8	(p<0.05)
RBC-GPX (U/g Hb)	: 38.5 ± 4.4	(p<0.01)

table II

DIETARY INTAKE
(at the entry)

	group 1 (23)	group 2 (21)
Total energy (kcal)	2890±344	3015±542
non-alcohol energy (kcal)	2380±253	2516±230
Protein (%)	15.2±2.5	15.7±3.0
Carbohydrate (%)*	57.3±9.8	54.9±7.9
Fat (%)	22.1±5.0	29.8±4.6
vitamin A (μg)**	1448±868	1698±993
vitamin E (mg)	8.3±2.7	8.4±2.4
vitamin C (mg)	78.5±28.9	81.2±43.2

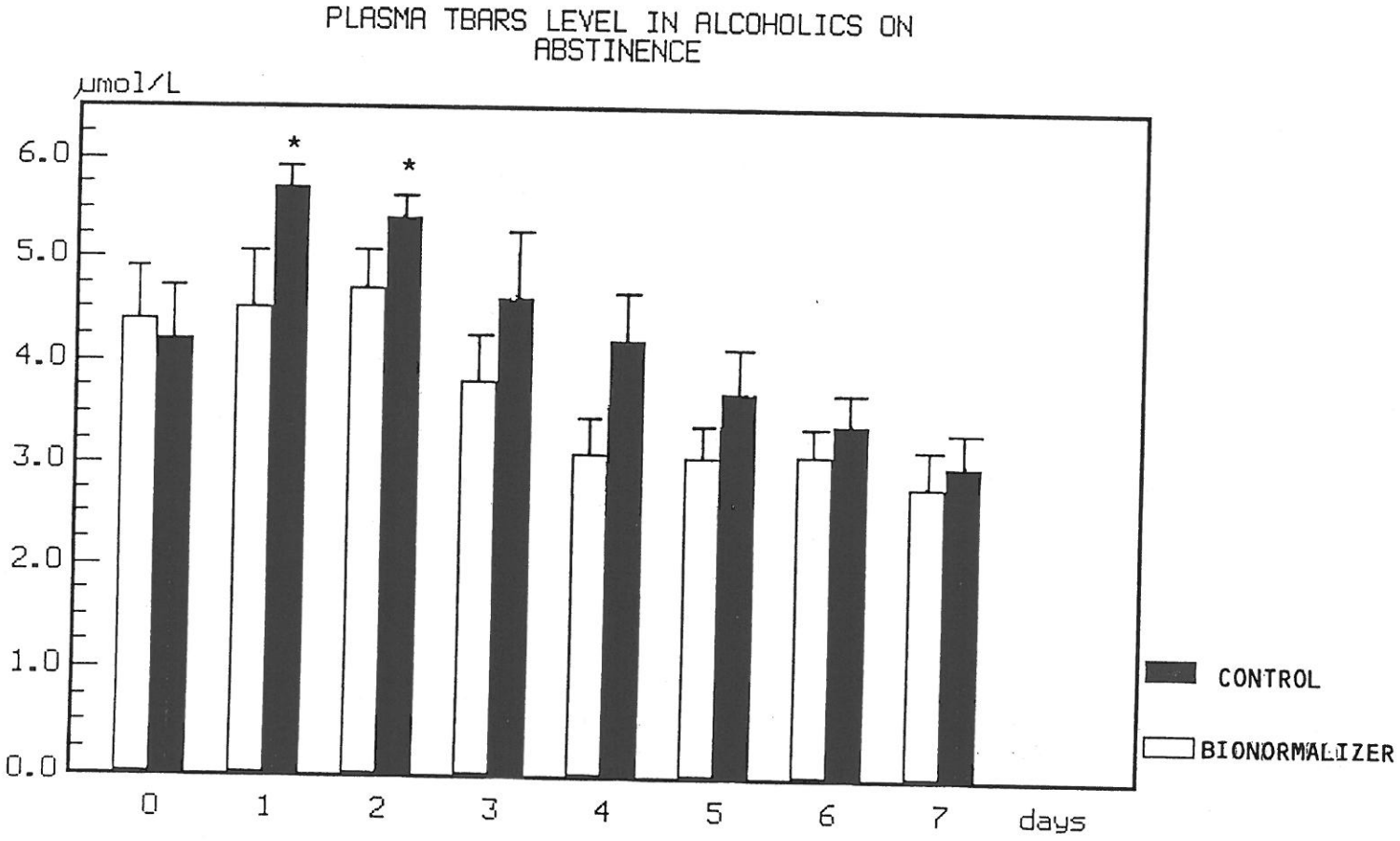
* percentage of non-alcohol energy.
** μg equivalent retinol = retinol (μg) + carotene (μg)/6



Time course changes of plasma lipid hydroperoxide level in alcoholics on abstinence and with or without Bionormalizer treatment.

$p < 0.01$ vs. control group.

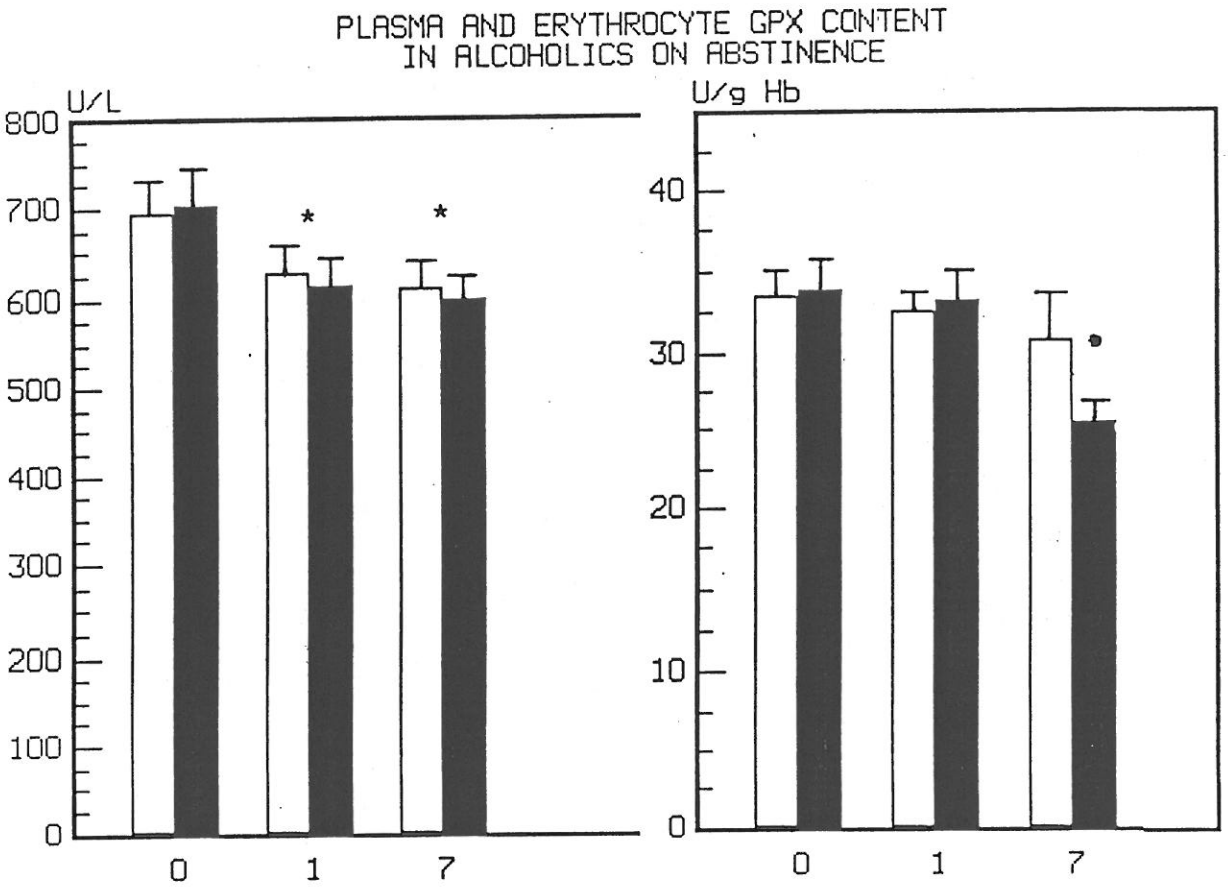
Fig 1



Time course changes of plasma TBARS in alcoholics with and without treatment with Bionormalizer while on abstinence.

* $p < 0.01$ vs. Bionormalizer group.

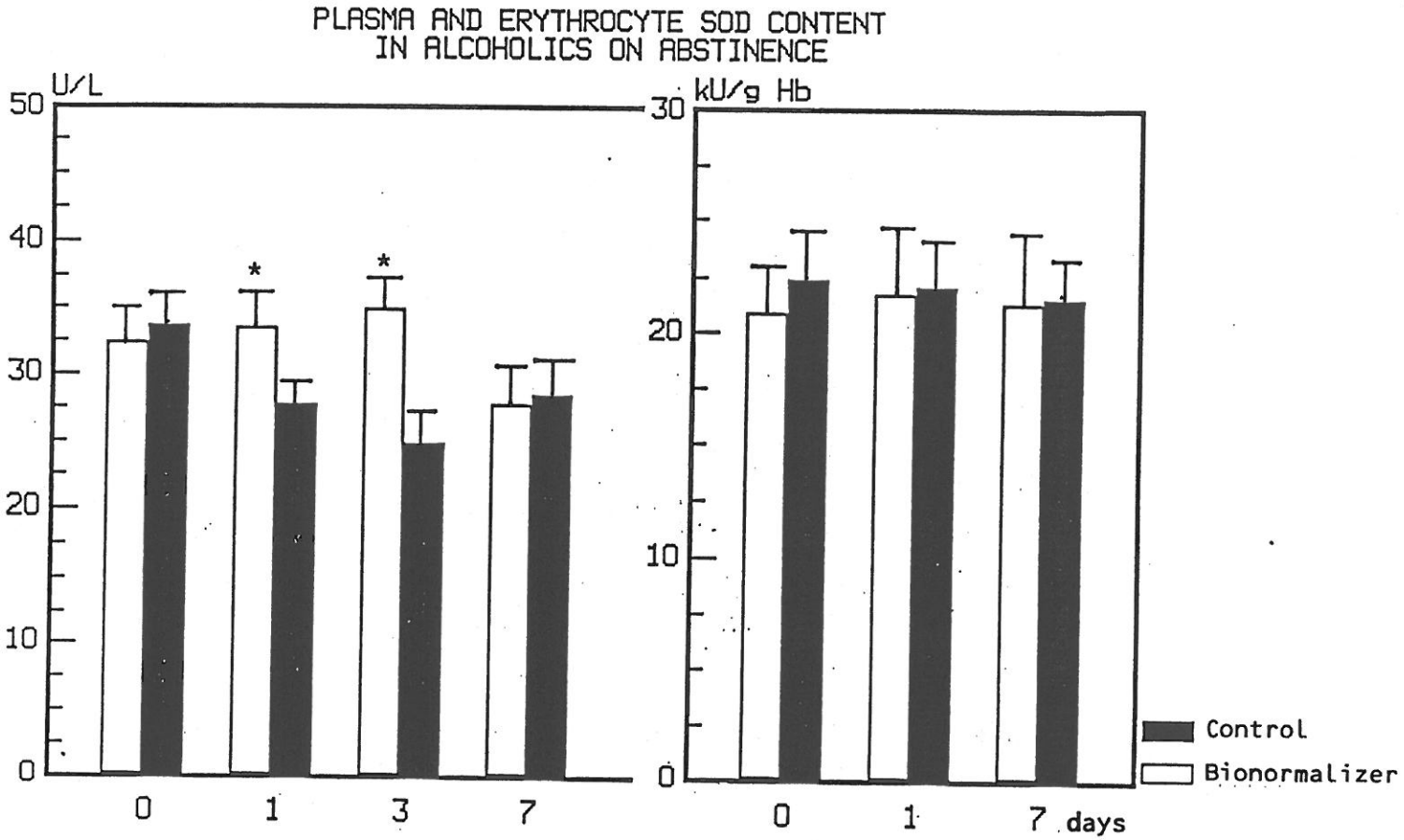
FIG 3



Time – course changes of plasma and erythrocyte GPX content in alcoholics on abstinence and with or without Bionormalizer treatment.

* $p < 0.05$ vs. pre-withdrawal level;

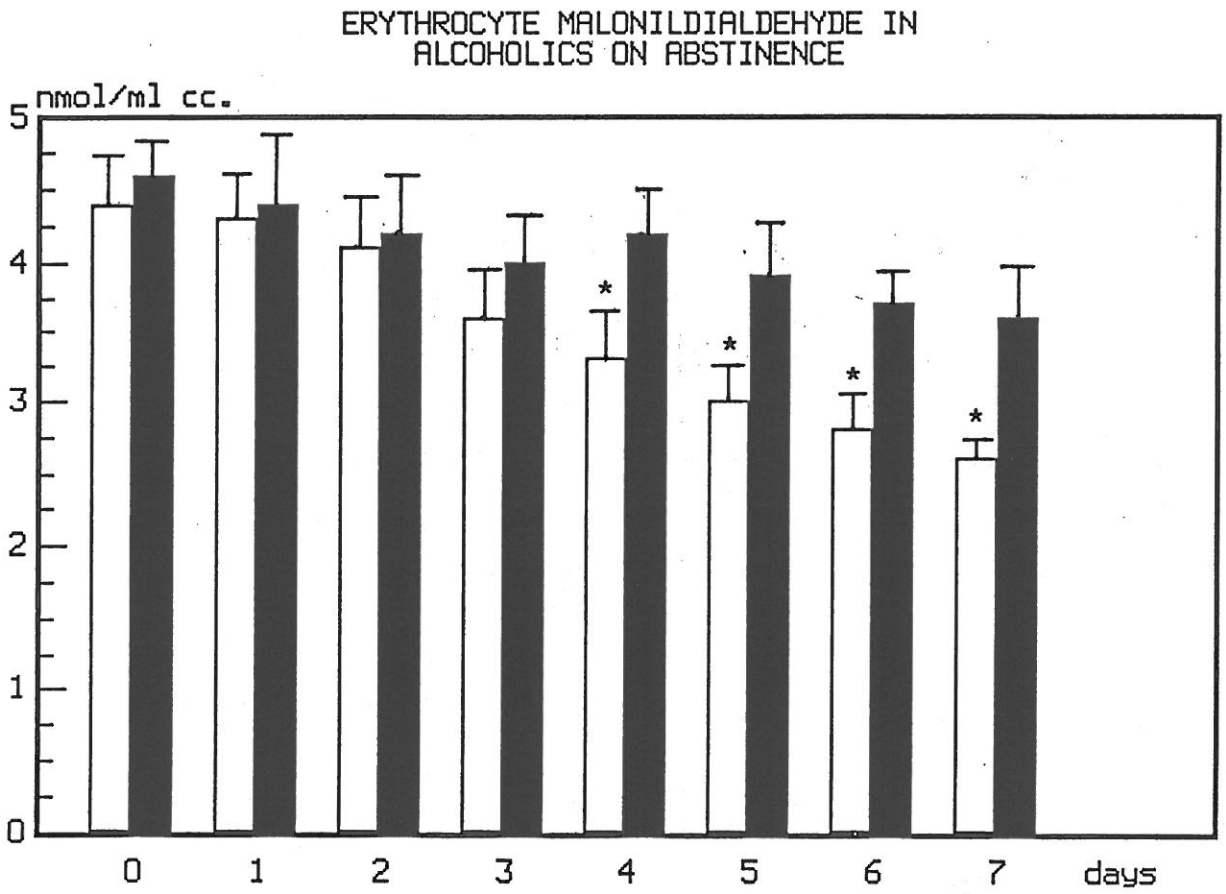
• $p < 0.05$ vs. Bionormalizer group.



Time – course changes of plasma and erythrocyte SOD level in alcoholics on abstinence and with or without Bionormalizer treatment.

* $p < 0.05$ vs. control group.

FIG 2



Time - course changes of erythrocyte malonildialdehyde content in alcoholics on abstinence with or without Bionormalizer treatment.

* $p < 0.05$ vs. control.